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13. ABSTRACT (Maximum 200) Interaction of the estrogen receptor with its ligands is mediated by a C-terminal region of the protein designated the hormone binding domain (HBD) and residues within the HBD are thought to contribute to dimerization. To examine dimer interactions in the isolated HBD, a human estrogen receptor HBD fragment was expressed in high yield as a cleavable fusion protein in <i>Escherichia coli</i> . The isolated HBD peptide exhibited high affinity binding for estradiol ($K_d \sim 0.2$ nM), binding stoichiometry of 1 mol estradiol/mol HBD, and cooperative estradiol binding with a Hill coefficient of ~ 1.6 . HBD dimer dissociation was found have a half-life of ~ 1.2 , which ligand binding increased ~ 3 -fold (estradiol) to ~ 4 -fold (4-hydroxytamoxifen). HBD constructs with altered N-termini exhibited differences in concentrations required for cooperativity and in dissociation half-life. These results suggest that the isolated estrogen receptor HBD dimerizes and undergoes conformational changes associated with cooperative ligand binding in a manner comparable to the full-length protein, that the N-terminus of the HBD contributes to dimer interactions, and that one effect of ligand binding is to alter the dissociation kinetics of the receptor protein dimer.				
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Larry E. Fisher 9-27-86
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INTRODUCTION

The estrogen receptor is a member of a superfamily of nuclear proteins that includes the receptors for the steroid hormones, for vitamins A and D, and for thyroid hormone (1,2). The binding of ligands to these receptors is the initial step in a complex series of events culminating in an interaction of the ligand-bound receptor with the transcription machinery and modulation of gene expression. These receptor proteins exhibit four distinct properties required to exert their actions: hormone binding, multimeric complex formation, sequence specific DNA binding, and transcriptional modulation. The currently proposed schematic structure of these receptor proteins (shown in Fig. 1 for the estrogen receptor), based on sequence similarities and deletion analyses (summarized in reference 2), suggests that these proteins fold into at least three separate structural and functional domains: (i) an N-terminal domain having a highly variable length and amino acid sequence and believed to mediate much of the transcriptional enhancement activity of the protein, (ii) a highly conserved central domain of ~80 amino acids involved in DNA-binding, and (iii) a less well conserved C-terminal domain of ~250 amino acids that is involved in ligand binding.

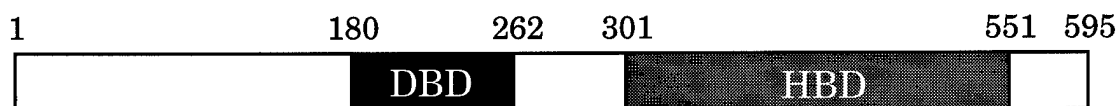


Figure 1. Schematic structure of the estrogen receptor. The locations of the N-terminal domain, DNA-binding Domain, and Hormone Binding Domain are shown using the amino acid numbering for the human protein.

The C-terminal hormone binding domain (HBD)¹ of the receptor is of particular interest because, in addition to its ligand binding activity, it appears to contain many of the regulatory functions of the protein. Chimeric constructs containing fusions of fragments of the estrogen receptor with unrelated proteins such as the *myc* oncogene product, for example, display hormonal regulation of the activity of the fused gene products (3). This suggests that, even when removed from its normal environment, the HBD is not only capable of specific ligand binding, but may also retain the capacity to undergo the conformational changes that normally regulate the function of the receptor. Furthermore, the finding that the HBD can affect the activity of unrelated proteins suggests that the ligand-induced alteration in conformation may be a fundamental change in structure. The structure of the estrogen receptor HBD has not yet been determined, but crystal structures of the HBD from the human retinoid-X-receptor (RXR- α), human retinoic acid receptor (RAR- γ), and rat thyroid hormone receptor (TR- α) have recently been solved (4-6). Comparison of the ligand-free RXR- α and the ligand-bound RAR- γ structures led to the suggestion that ligand binding to the nuclear-receptor family proteins alters the conformation of the C-terminus of the HBD, a region previously proposed to contain a conserved transcriptional activation function (7,8).



Figure 2. Sequence alignment of human estrogen receptor HBD with human RXR-α HBD (after Wurtz *et al.* (8); another alignment of the N-terminal ~40 residues (equating RXR Ser-225 with Ser-305 of ER, rather than Ser-309) is possible which yields a smaller gap around hER residue 331 with the same number of identical residues). The residues visible in the RXR crystal structure are shown for comparison. Dashes in the RXR sequence indicate identities with the ER. The numbers between the sequences show the locations of the α-helices in the RXR structure (bold indicates agreement with secondary structure prediction for the ER); the vertical lines indicate the β-sheet in the RXR. The α symbols indicate the three ER HBD tryptophan residues (note that Trp-393 is not conserved in the RXR sequence). The Δ symbols indicate important dimer contacts in the RXR structure.

The nuclear receptor superfamily proteins are thought to form dimers. This property has at least two functional roles: most of the proteins in the family are thought to bind DNA as dimers, and dimer formation allows cooperative ligand binding, thereby narrowing the ligand concentration range required for full biological effect. The nature of the dimer interface of the full-length receptor protein is not established. The isolated DNA-binding domain has been shown to dimerize in the presence of DNA, suggesting that some of the dimerization interface resides within this portion of the protein; the isolated DNA-binding domain, however, is monomeric in solution. The HBD is also thought to play a role in dimerization. Mutations of residues within the estrogen receptor HBD have been shown to inhibit dimer formation (9). The RXR-α HBD crystallized as a dimer; however, the ligand-bound RAR-γ HBD and TR-α HBD crystallized as monomers. This raises the question as to how much of the interprotein interaction involved in dimer formation is actually contained within the HBD and whether the isolated HBD is fully capable

of cooperative ligand binding. Although the RXR, RAR, and TR are clearly evolutionarily-related to the estrogen receptor, their HBDs exhibit less than 25% sequence identity to that of the estrogen receptor (see Figure 2 for sequence alignment of estrogen receptor HBD with RXR- α). Moreover, the estrogen receptor is thought to form exclusively homodimers in contrast to the capacity for heterodimer formation by the RXR, RAR, and TR. Thus, there are likely to be structural and functional differences between the estrogen receptor and the proteins for which three dimensional structural information is available.

Characterization of the estrogen receptor HBD has been limited by the difficulty of obtaining sufficient amounts of the peptide. In order to obtain preparations of the estrogen receptor HBD for more detailed study, several groups have attempted to express peptides containing the HBD in heterologous systems. Expression of the estrogen receptor HBD in yeast and bacteria has generally resulted in low yields of protein: ≤ 1 mg/liter (10,11). Recently, Seielstad *et al.* (12) reported expression of an isolated HBD fragment in high yields in *E. coli*; however, the protein produced using this system was insoluble, necessitating the use of urea during purification and characterization.

Current progress includes determination of ligand binding stoichiometry, development of an assay for examining dimer dissociation kinetics in solution, and construction and screening of several mutant proteins.

List of abbreviations

The abbreviations used are: AEBSF, [4-(2-aminoethyl)-benzenesulfonylfluoride]; CD, circular dichroism; DEAE, diethylaminoethyl; DTT, dithiothreitol; HBD, hormone binding domain; MBP, maltose-binding protein; RAR, retinoic acid receptor, RXR, retinoid-X-receptor; TED, Tris-EDTA-DTT, TR, thyroid hormone receptor.

MATERIALS AND METHODS

Supplies – Restriction endonucleases and other enzymes used for DNA manipulation were obtained from Boehringer-Mannheim Corp. (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), Stratagene Cloning Systems (La Jolla, CA), or United States Biochemical Corp. (Cleveland, OH). Synthetic oligonucleotides were obtained from Operon Technologies (Alameda, CA). Bacterial growth media components were purchased from Difco (Detroit, MI); other reagents were obtained from Sigma Chemical Company (St. Louis, MO). Tritiated estradiol was obtained from Amersham and New England Nuclear. The estrogen antagonist *trans*-4-hydroxytamoxifen was a gift from Dr. Dominique Salin-Drouin (Laboratoires Besins-Iscovesco) and ICI 182,780 was a gift from Dr. Alan Wakeling (ICI Pharmaceuticals).

Vector Construction – Unless otherwise noted, all DNA manipulations were carried out by standard techniques (13). As described in the previous progress report, a DNA fragment coding for the human estrogen receptor hormone binding domain (amino acids 301-551) was generated by PCR from the HE0 estrogen receptor cDNA plasmid (14) using the following primers: 5' primer TCTAAGAAGAACAGCCTGGCCTTG, and 3' primer atcCgAaTtcaCGCATGTAG GCGGTGGGCGTCCAG; lowercase bases in the 3' PCR primer are mismatches that convert the codon for Pro-552 to a TGA termination codon and create an *Eco*RI site for sub-cloning. The PCR fragment was digested with *Eco*RI and sub-cloned into the pMAL-c2 vector (New England Biolabs) which had been digested with *Xmn* I and *Eco*RI. Following isolation of the insert-containing plasmid, the entire HBD coding region was sequenced to confirm the absence of errors introduced by PCR amplification. The presence of the cDNA mutation Gly400Val (15) was verified by DNA sequencing; this mutation was reverted to wild-type using a PCR mutagenesis procedure (16), creating the plasmid pER08 (the Appendix contains a list of plasmids and their designations).

Protein products of pMAL-c2 derived plasmids consist of the maltose binding protein fused to the desired protein with a linker peptide consisting of (Asn)₁₀-Leu-Gly-Ile-Glu-Gly-Arg; the terminal four residues of the peptide comprise a Factor X_a cleavage signal. Factor X_a hydrolysis of the expressed fusion protein, however, resulted in heterogeneous, largely inactive peptides; we therefore modified the linker region to generate the sequence Asn-Gly, which can be cleaved by hydroxylamine (17). Bases encoding residues Leu-Gly-Ile-Glu of the Factor X_a recognition sequence were mutated to Asn codons by site-directed mutagenesis using the unique site-elimination procedure (18) with the Transformer kit from Clontech (Palo Alto, CA). The coding region of the mutagenesis product was sequenced; the modified DNA was found to encode a linker peptide of (Asn)₁₄-Gly-Arg. This plasmid was designated pER304. Unique site-elimination was then performed on pER304 to mutate Ser-305 to Glu, creating pER336. The product of hydroxylamine cleavage of the fusion protein from pER304, pER336, and a number of constructs derived from these plasmids retains Gly-Arg from the linker, the latter of which corresponds to the naturally occurring Arg-300.

Plasmids beginning at sites other than position 300 were constructed by sub-

cloning PCR products into pMAL-c2, or into pMAL-INGR (pMAL-c2 in which the Glu from the Factor X_a site was mutated to Asn) in a manner similar to that described for pER08. Other plasmids discussed were constructed by site-directed mutagenesis of previously existing plasmids containing appropriate characteristics.

Protein Expression and Purification – Competent TOPP2 cells (Stratagene) were transformed with the expression plasmids. Cells containing the appropriate plasmid were grown in TB media in the presence of 100 µg/ml ampicillin to an OD₆₀₀ of ~1.7; protein expression was induced by the addition of IPTG to a final concentration of 0.25 mM and cultures were grown overnight at ambient temperature (usually ~27 °C).

For screening assays, the cells were harvested, lysed essentially as described below, and the lysate supernatant used directly in the radioreceptor assay. For purification, cells were harvested by centrifugation and frozen overnight at -20 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 2 mM DTT, 1 mM AEBSF (Cal Biochem), pH 8.0, and 1 mg lysozyme/g of cells). After ~1 hr at ambient temperature, MgCl₂ was added to a final concentration of 120 mM and the lysate treated with DNase and RNase. The supernatant from a 40,000 x g centrifugation of the lysate was diluted 3-fold in TED buffer (20 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT, pH 7.3) and applied to a DEAE-cellulose column (Whatman). The flowthrough from the DEAE-cellulose column was applied to an amylose resin column (New England Biolabs). After washing with 2-4 column volumes of TED containing 0.2 M NaCl, the fusion protein was eluted with 10 mM maltose in the same buffer.

The eluted protein was diluted 5-fold and applied to a DEAE-Sepharose column (Pharmacia). This column was washed with 5 column volumes of TED containing 0.05 M NaCl, and the protein was eluted with a linear NaCl gradient (0.05-0.2 M NaCl); the fusion protein eluted at 0.13-0.16 M NaCl. The fusion protein was then concentrated to ~20 mg/ml by precipitation with 60% ammonium sulfate and was digested for 60-72 hours at ambient temperature with hydroxylamine (final concentration: 2 M hydroxylamine-HCl, 0.2 M Tris-HCl, pH 9.0). The cleaved HBD peptide was separated from the maltose binding protein by Sephadex G-100 gel filtration chromatography. The final preparation of the purified HBD peptide was stable and could be stored at 4 °C or -70 °C for several months.

As discussed in the Results section, early preparations of the fusion protein and of the HBD had apparent estradiol binding stoichiometries significantly lower than 1:1, although the other properties of the protein were similar to those reported here. Addition of the DEAE-Sepharose chromatography step to the purification procedure for the fusion protein raised the stoichiometry of estradiol binding to close to 1:1, although this step had little effect on the apparent purity as assessed by SDS-PAGE. In contrast, FPLC Superdex-200 or gravity Sephadex G-100 gel filtration chromatography had no effect on the activity of the fusion protein or HBD samples.

Spectroscopy – All spectroscopy was performed at ambient temperature. Absorbance spectra were obtained using a Cary 1 spectrophotometer calibrated with K₃Fe(CN)₆ assuming $\epsilon_{420} = 1,020 \text{ (M}\cdot\text{cm)}^{-1}$. The concentration of purified MBP-

HBD fusion protein and of isolated HBD peptide were determined spectrophotometrically assuming $\epsilon_{280} = 89,365 \text{ (M}\cdot\text{cm)}^{-1}$ for the fusion protein and $23,745 \text{ (M}\cdot\text{cm)}^{-1}$ for the HBD peptide; these values are based on a composition of 11 tryptophan and 20 tyrosine residues (fusion protein) or 3 tryptophan and 5 tyrosine residues (HBD peptide) predicted from the cDNA sequence and on average extinction coefficients for tryptophan ($5615 \text{ (M}\cdot\text{cm)}^{-1}$) and tyrosine ($1380 \text{ (M}\cdot\text{cm)}^{-1}$) (19,20). Concentrations of HBD determined spectrophotometrically agreed closely with those determined by the methods of Lowry *et al.* (21) and Bradford (22).

Analytical Gel Filtration – The apparent molecular weight of the fusion protein and HBD were determined using a Pharmacia FPLC system and a Superdex 200 HR 10/30 gel filtration column (running buffer 20 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.3). The column was calibrated using blue dextran to determine the void volume and with the following standard proteins: thyroglobulin (669 kDa), ferretin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (69 kDa), ascorbate peroxidase (57.5 kDa), P450eryF (45.8 kDa), ovalbumin (43 kDa), MBP (40.4 kDa), rhodanese (33.3 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa), and cytochrome c (12.4 kDa).

For the kinetic experiments, equimolar amounts of the fusion protein and HBD peptide were mixed and incubated at ambient temperature ($\sim 25^\circ\text{C}$). At various times aliquots were taken and subjected to FPLC gel filtration. For the experiments in the presence of ligand, the column was pre-equilibrated in the same running buffer with 50 nM of the relevant ligand, and 2 μM solutions of each protein pre-equilibrated overnight with 5 μM of the ligand. The integrated peak areas were corrected for extinction coefficient of the relevant protein species to determine the concentration of each species (*i.e.* fusion homodimer, HBD homodimer, or heterodimer) present at the time of injection (the relative amount of each species was assumed not to change during the chromatography). For experiments in the presence of ligand, the extinction coefficient of the protein was corrected for contributions of the bound ligand (assumed to be $\sim 2,000 \text{ (M}\cdot\text{cm)}^{-1}$ for estradiol and $\sim 15,000 \text{ (M}\cdot\text{cm)}^{-1}$ for 4-hydroxytamoxifen).

The rate constant for dissociation, k , was determined by least-squares non-linear regression of the first order rate equation:

$$D_t = (D_0 - D_f)e^{-kt} + D_f$$

where D_t is the concentration of one homodimer at time t , D_0 is the initial concentration of homodimer, and D_f is the final concentration of homodimer after the rearrangement had gone to completion. Half-life ($t_{1/2}$) for dissociation is defined as $\ln(2)/k$.

Protein Sequence Determination – Amino-terminal sequence data was obtained for purified cleaved protein by automated Edman degradation performed by Dr. Agnes Henshen-Edman (University of California, Irvine).

Radioreceptor Assay – The HBD peptide was incubated overnight with various concentrations of $[6,7\text{-}^3\text{H}]$ -estradiol at 4°C in TED buffer including 0.2 M NaCl and

1 mg/ml porcine gelatin; bound and unbound steroids were separated using dextran-coated charcoal (0.625% charcoal, 0.125% dextran) in the same buffer without gelatin. In all experiments using purified and partially purified protein, the binding of radioactive estradiol in the presence of a 100-fold excess of unlabeled estradiol was equivalent to the non-specific binding observed in the absence of any added HBD protein. The presence of a carrier protein in both the ligand and protein buffers was found to be necessary to obtain reproducible results; porcine gelatin (1 mg/ml), bovine γ -globulin (4 mg/ml), or bovine serum albumin (4 mg/ml) gave similar results.

The data for bound and free steroid were directly fitted to the Hill equation (24):

$$[B] = \frac{B_{\max} [F]^n}{(F_{0.5})^n + [F]^n}$$

using least squares non-linear regression analysis to estimate the $F_{0.5}$ (or K_d when $n = 1$), B_{\max} , and n (Hill coefficient).

RESULTS AND DISCUSSION

Stoichiometry of Ligand Binding

At the time of the last progress report, we had reproducibly obtained ligand binding corresponding to a stoichiometry of 0.5 mol estradiol/mol HBD peptide. More recent experiments suggest that this conclusion was incorrect, and that the true value is probably 1 mol estradiol/mol HBD peptide. Although a number of additional purification steps for previous preparations did not improve the activity of the protein, a modification to the purification procedure increased the apparent ligand binding by ~2-fold.

The pMAL-c2 expression system used in these studies produces the protein of interest as a fusion with the *E. coli* maltose binding protein. The MBP is well expressed, stable, and can be purified by amylose affinity chromatography. In the commercial system, the linker peptide of the fusion protein is designed to be cleavable by the endoproteinase Factor X_a to release the fused protein without additional N-terminal residues. Attempts to purify MBP-HBD fusion proteins from crude cell homogenates by amylose affinity chromatography, however, were unsuccessful; binding of the fusion protein from the crude extract to the affinity column was incomplete, and the eluted protein was not highly purified. As a result, the protein was subjected to DEAE-cellulose anion exchange chromatography, either with or without prior ammonium sulfate fractionation, and the eluent subjected to the amylose column. Under these conditions, the protein bound essentially quantitatively. However, following elution from the amylose column, the apparent stoichiometry of binding was ~0.5 mol/mol in nearly all experiments. Gel filtration chromatography had no effect on the observed stoichiometry.

Although anion exchange chromatography had been used earlier in the procedure, the fusion protein was then subjected to DEAE-Sepharose chromatography. The bound protein was washed with several column volumes of TED buffer, and the protein eluted with a linear sodium chloride gradient. Although the apparent homogeneity as assessed by SDS-PAGE did not appear to change, the stoichiometry of binding in the radioreceptor assay increased markedly. After this protein was treated with hydroxylamine, the isolated HBD also exhibited a stoichiometry of ~1 mol estradiol/mol HBD (Fig. 3).

The current purification protocol, given in the Methods section in more detail, subjects the lysate supernatant to DEAE-cellulose chromatography (the ammonium sulfate step is no longer necessary, which decreases the time required for the procedure and increases the yield of protein). The fusion protein does not bind to the DEAE-cellulose column under the conditions used; the unbound material is then applied to the amylose column. The eluent from the amylose column is then subjected to DEAE-Sepharose chromatography. The remainder of the procedure is similar to that described in the previous progress report.

The reason for the increased stoichiometry is not understood; it may be due to removal of small molecular weight contaminants or to reduction of disulfide bonds

in the protein by the DTT in the TED buffer (it is thought that the Cys residues within the HBD are reduced in the active protein).

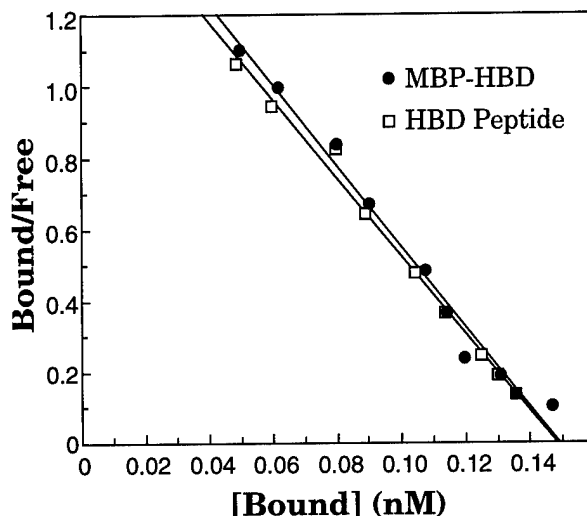


Figure 3. Scatchard analysis for estradiol binding to the MBP-HBD peptide fusion protein and purified HBD peptide (the proteins used are derived from the pER336 plasmid; similar results were obtained from several other plasmids. A radioreceptor assay was performed using 0.15 nM MBP-HBD peptide protein or 0.15 nM purified HBD peptide. Both non-linear regression analysis of the bound and free data, and linear regression of the bound/free *versus* bound Scatchard transformation data yield apparent K_d values of 0.1 nM and apparent B_{max} values corresponding to 0.98 mol estradiol/mol protein for both the fusion protein and the HBD peptide.

It was obviously necessary to repeat the binding experiments using the more active preparations of the HBD protein. In doing so, no significant difference were observed, suggesting that the source of the discrepancy in binding stoichiometry did not affect the observable properties of the protein.

Fusion Protein Cleavage

Following expression and purification of the fusion protein, the ER HBD peptide must be released from the fusion and isolated. The commercial pMAL-c2 vector is intended to produce fusion proteins cleavable with Factor X_a . We were unsuccessful in obtaining homogeneous cleavage with this enzyme, and turned to hydroxylamine cleavage as an alternative. Although hydroxylamine preferentially cleaves Asn-Gly sequences, it is also capable of cleaving exposed Asn-Xaa sequences. This somewhat limited specificity has caused some difficulty in this project.

The initial "wild-type" hydroxylamine-cleavable construct, pER304, is intended

to contain an Asn-Gly immediately upstream of Arg-300. As described in the previous progress report, although the majority of the peptide did, in fact, cleave at the intended site, a significant minority of the peptide cleaved between Asn-304 and Ser-305 of the HBD sequence.

pER08	NSSSNNNNNNNNNNLGIEGRSKKNSLALS...551
pER304	NSSSNNNNNNNNNNNNN NG RSKKNSLALS...551
pER330	NSSSNNNNNNNNNNLG ING RSKSLALS...551
pER331	NS SSNNNNNNNNNNNLGIEGREVGSAGDMRAANLWPSPLMIKRSKK NG LALS...551
pER333	NS SSNNNNNNNNNNNLGIEGRSKK NG LALS...551
pER334	NS SSNNNNNNNNNNNLG ING RSKSLALS...534
pER335	GS SSNNNNNNNNNNNLGIEGRSKK NG LALS...551
pER336	NSSSNNNNNNNNNNNNN NG RSKKNELALS...551

Figure 4. Plasmids with different cleavage sites for hydroxylamine. Immediately preceding the NSSS sequence shown at the left for each construct are amino acids 1-367 of the maltose binding protein. The sites known or suspected to be cleaved by hydroxylamine are shown in bold for each construct. The sequences intended to become part of the final HBD peptide are underlined. not designed to be hydroxylamine cleavable.

Three approaches were tested during attempts to generate homogeneously cleavable fusion proteins. The first approach involved constructing a plasmid that lacks the Asn-Ser cleavage site. The plasmid pER330 (Fig. 4), constructed by subcloning a PCR fragment into pMAL-INGR (a modified pMAL-c2 vector; note the INGR instead of IEGR sequence at the cleavage site); it is intended to produce a peptide that includes Gly-Arg in addition to amino acids 305-551 of the estrogen receptor. The fusion protein expressed by pER330 cleaves essentially quantitatively at the correct site as assessed by SDS-PAGE (Fig. 5) and by N-terminal sequence analysis (data not shown). However, differences in the behavior of this peptide compared to the pER304 derived HBD peptide (mentioned in the previous Progress report, and discussed in Cooperativity of Estradiol Binding section (see below), suggested that other approaches were necessary.

The second approach was to mutate the pER08 Ser-305 to Gly, creating pER333, which contains an Asn-Gly sequence that should be the primary cleavage site. Upon cleavage of the pER333 fusion protein, however, two protein bands appeared on the SDS-PAGE (Fig. 6).

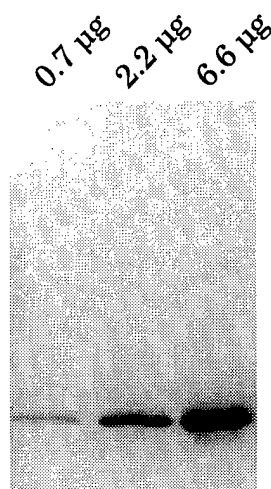


Figure 5. SDS-PAGE for purified pER330 HBD (Gly-Arg+305-551) peptide.

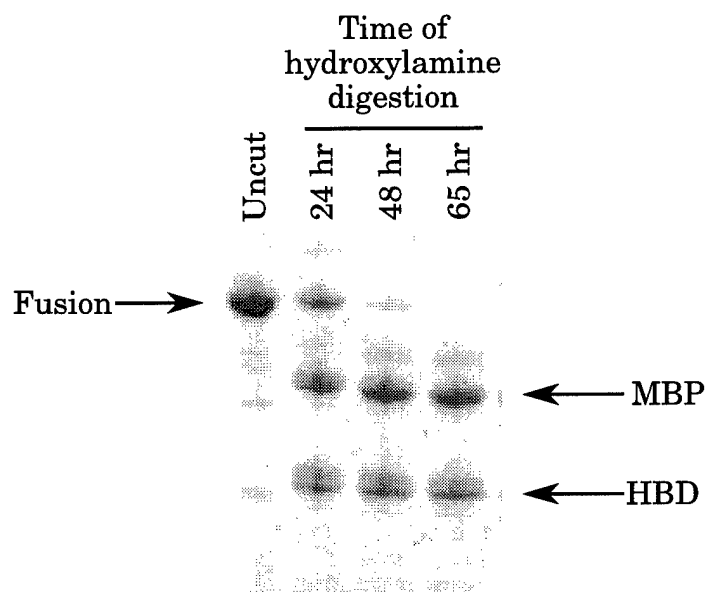


Figure 6. Phast gel SDS-PAGE on pER333 fusion protein. Note second protein band immediately above HBD.

The reason for the heterogeneous cleavage was not immediately obvious. The additional cleavage site produced a product that was >1 kDa larger than the HBD; this was therefore not the result of cleavage within the HBD. Testing of an additional S305G construct, pER331, revealed the source of the additional protein. The plasmid pER331 codes for estrogen receptor amino acids 279-551 (see Fig. 4); digestion of the pER331 fusion protein yielded a similar pattern to that for pER333 (Fig. 7).

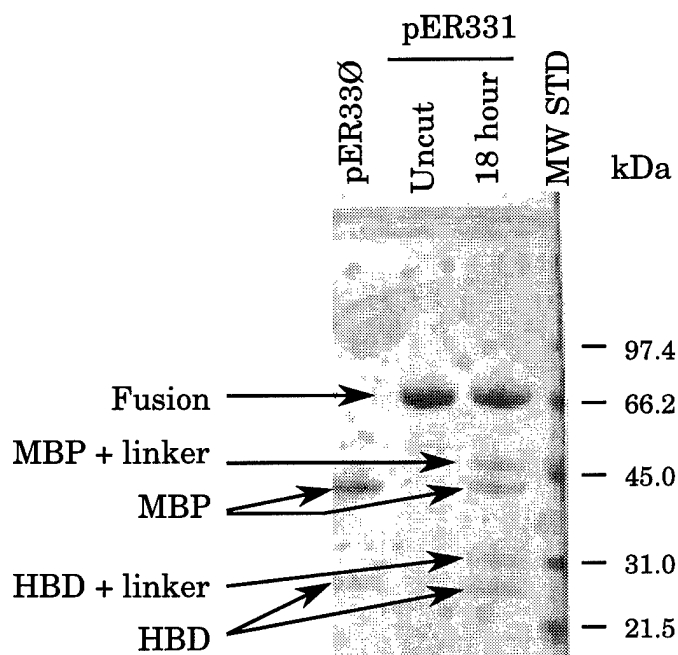


Figure 7. Phast gel SDS-PAGE for pER331. Note the additional band above the MBP and above the HBD. In each case the additional band is ~5 kDa larger than the correct size, corresponding to the addition of the linker+residues 279-304 (predicted size: 4882 Da).

Based on these results, it was concluded that an additional cleavage site was present at the N-terminal side of the linker. The most likely site is the Asn-Ser sequence that precedes the poly-Asn linker peptide, although mutation of this Asn to Gly (to create pER335) did not prevent formation of the additional band (Fig. 8). It is possible that the pER335 peptide is being cleaved within the poly-Asn sequence, since there are no other Asn residues in locations that could account for the observed sizes of the bands on the gel.

The third approach to forcing cleavage at the correct site is to mutate either the Asn-304 or the Ser-305 to prevent cleavage at the incorrect site. The plasmid pER336 contains a Ser-305 to Glu mutation. This construct cleaves at a single site (Fig. 9).

During the cleavage reaction, the HBD peptide generally precipitates. There are two reasons for this behavior: 1) the HBD peptide is less soluble following release from the MBP, and 2) for most preparations, the fusion protein was concentrated using ammonium sulfate precipitation; although the fusion protein is soluble in the presence of the residual ammonium sulfate following resuspension of the pellet, the released HBD peptide is less soluble, and precipitates. The majority of the HBD peptide can be solubilized and remains active, although some of the protein appears to become denatured. For plasmids in which multiple cleavage sites exist, it is possible to alter the ratio of cleavage between the site closer to the HBD and the site closer to the MBP by concentrating the fusion protein using ultrafiltration rather than ammonium sulfate (or by dialyzing to remove the ammonium sulfate after

precipitation). Use of this technique has allowed production of HBD peptide from plasmids such as pER335 with greatly reduced (although not abolished) cleavage at the incorrect site.

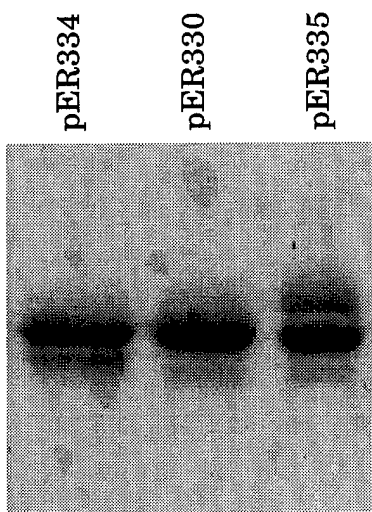


Figure 8. SDS-PAGE for cleaved HBD peptides from three constructs. The pER330 peptide appears to have cleaved at a single site. The pER335 peptide appears to have cleaved at two sites, to yield a peptide of similar size to the pER330 peptide (predicted cleavage sites yield 249 amino acids for pER330 and 247 amino acids for pER335), and a somewhat larger peptide (~2.5 kDa larger, corresponding to the size of the linker peptide).

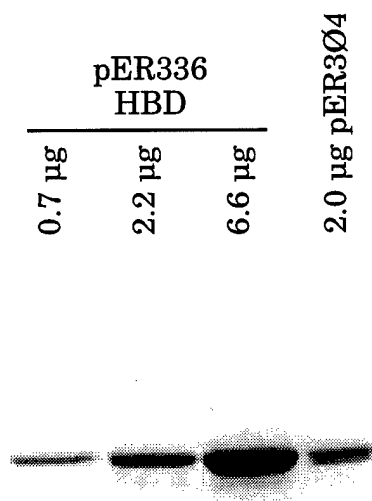


Figure 9. SDS-PAGE for isolated pER336 HBD (Gly+300-551, S305E) and for pER304 (Gly+300-551). The pER336 HBD was cleaved with hydroxylamine and separated from the MBP by Sephadex G100 chromatography; the pER304 HBD was similarly treated, and then re-treated with hydroxylamine, and re-chromatographed in an attempt to obtain a homogeneous preparation.

There are some limits to the ability to direct cleavage using this technique, however. It appears the cleavage at one site may alter the conformation of the N-terminus of the HBD peptide such that the remaining site (if still contained within the HBD peptide) is much less vulnerable to cleavage. One of the pER304 HBD peptide preparations was subjected to a second hydroxylamine cleavage reaction. The relative amount of HBD peptide cleaved at the internal site (*i.e.* between Asn-304 and Ser-305) remained less than 50% in spite of 67 hours of exposure to hydroxylamine (see Fig. 9), suggesting that the N-terminal region has assumed a conformation with insufficient flexibility to allow cleavage to occur.

Conformation induced interference with the cleavage reaction was also observed with the fusion protein expressed from the pER334 plasmid. The sequence of this construct is identical to pER330 in the region surrounding the cleavage site; the sole difference is the replacement of the codon for Pro-535 with a stop codon by site-directed mutagenesis. This should result in a peptide 16 amino acids (~2 kDa) smaller than that produced by pER330. As can be seen in Fig. 8, the major HBD band following hydroxylamine cleavage is very similar in size to that of the pER330 product; only the minor band is of the expected size. This suggests that the altered structure of C-terminus induced by the deletion of the last 16 amino acids changes the conformation of the cleavage site and decreases the probability of cleavage at the correct site.

Size Exclusion Chromatography

The hormone binding domain is thought to contain a region at least partially responsible for dimerization of the full-length protein (2,9,29). Gel-filtration chromatography was used to determine whether the fusion protein and HBD peptide formed dimers (or larger multimers) in solution. For all of the constructs thus far tested, when the cleaved HBD peptide was separated from the MBP by Sephadex G-100 gel filtration chromatography during the purification procedure, the HBD peptide was found to elute near the void volume of the column, well ahead of the ~40 kDa MBP. This suggests that the HBD peptide (monomer ~29 kDa) exists as a multimeric complex under the conditions used for the preparative G-100 gel filtration (~100 μ M initial peptide concentration). This was examined further using analytical FPLC Superdex-200 gel filtration chromatography. When run independently on the Superdex column, both the fusion protein and HBD peptide migrated as single peaks. The apparent M_r of the fusion protein, 156 k, was similar to that predicted for a dimer (142 k). Because the isolated MBP is known to be monomeric and migrates close to its predicted size of 40 k, this suggests that fusion protein dimerization is mediated by the HBD peptide fragment. The HBD peptide alone migrated with an apparent M_r of 47 k, intermediate between that expected for a dimer (58 k) or monomer (29 k). To determine whether the HBD peptide exists as a dimer, equimolar amounts of the fusion protein and HBD peptide were mixed and then subjected to gel filtration ~24 hours later. The delay of ~24 hours was necessary to allow dimer dissociation and reassociation; as shown in Fig. 10, and discussed in more detail in the next section, this rearrangement is a rather slow process.

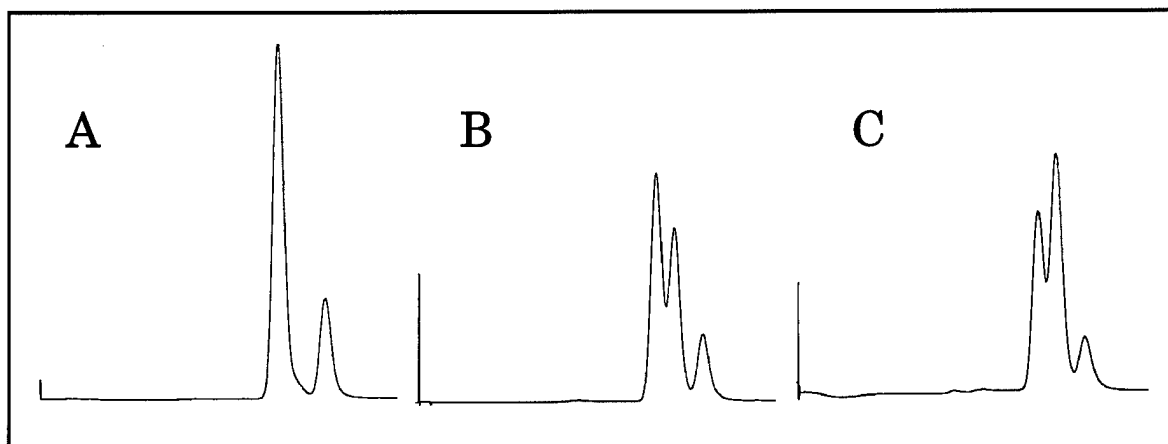


Figure 10. Change in the FPLC chromatogram with time after mixing equimolar amounts of pER336 fusion protein and pER336 HBD peptide. Panel A shows the results of an injection within 2 minutes after mixing; Panel B, after 3 hours, and Panel C, after 24 hours.

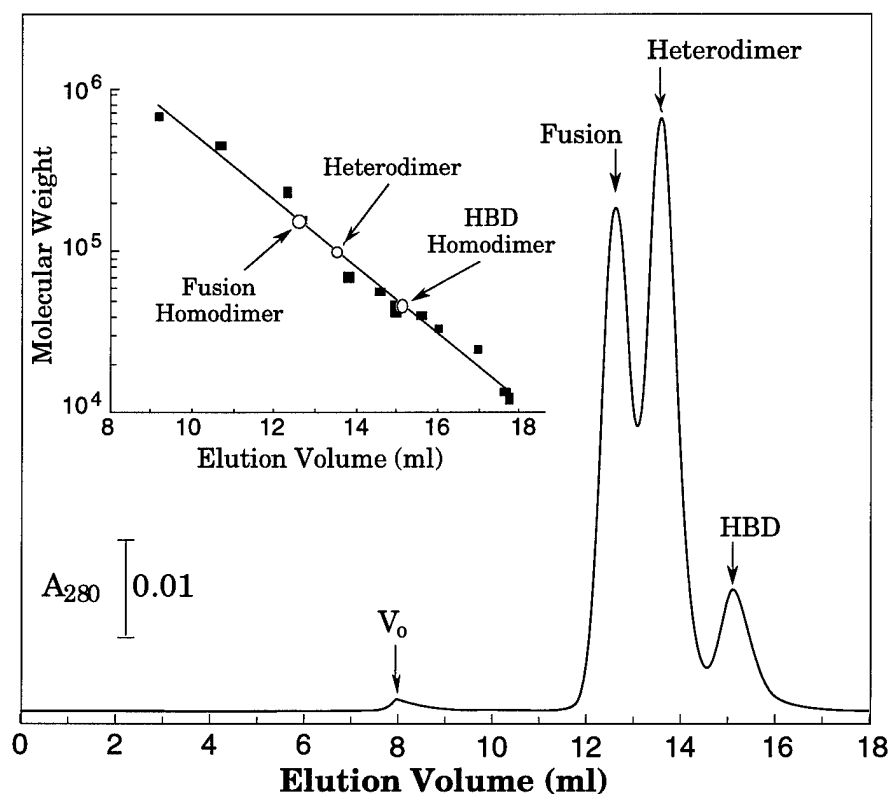


Figure 11. FPLC chromatogram of a mixture of fusion protein and HBD peptide (this experiment used pER336 derived peptides; similar results have also been observed for pER304, pER330, and pER335; other constructs have not yet been tested). The peaks labeled "fusion protein" and "HBD peptide" co-migrate with purified samples of the corresponding protein run separately; the peak labeled "heterodimer" only appears when the two proteins are mixed prior to chromatography. The experiment shown was performed in the absence of ligand; no change in migration was observed in the presence of estradiol or 4-hydroxytamoxifen (data not shown). The inset shows the migration positions of the estrogen receptor-derived protein complexes in comparison with the standard proteins used to calibrate the column (see Methods).

Following mixing, a third peak appeared at 99 k (Fig. 10 and 11), a position corresponding to a heterodimer of the fusion protein (72 k) and the HBD peptide (29 k). Integration of the peak areas (corrected for the extinction coefficients of fusion homodimer, heterodimer, and HBD peptide homodimer) yielded a ratio of 1:2:1, confirming the identity of the intermediate peak as a heterodimer formed between fusion protein and HBD peptide monomers. The fact that a single additional peak was formed also suggests that both fusion protein and HBD peptide predominantly form dimers, but not larger multimers, in solution. No significant changes were observed in the migration of the HBD peptide or fusion protein on the Superdex column using concentrations ranging from 0.5 to 10 μ M. This suggested that for both the fusion protein and HBD peptide, the majority of the protein was present as dimer under these conditions.

Kinetics of Dimer Dissociation

In order to form the heterodimer, these homodimers must dissociate, and as suggested by the fact that only dimers are detected in the gel-filtration experiment, this dissociation probably constitutes the rate limiting step for heterodimer formation. The rearrangement reaction is shown in Fig. 12. The measured rate of dissociation depends on the slower of the rates for dissociation of the HBD or fusion homodimer.

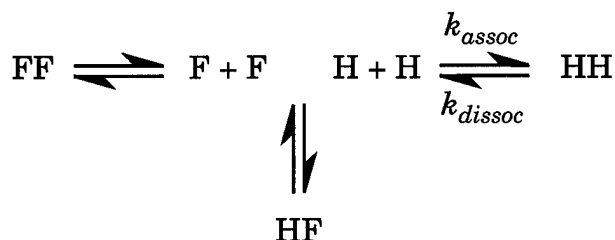


Figure 12. The dimer rearrangement reaction. FF, fusion protein homodimer; HH, HBD homodimer, HF, heterodimer. Since dimer formation is mediated by the HBD, and since the HBD sequence present in the fusion protein and HBD are identical in each experiment, it is likely that the k_{dissoc} , is similar for both fusion protein and HBD homodimers and for the heterodimer; if not, the measured value will be that of the slower of the two homodimer dissociation rates.

The fusion protein and HBD peptide expressed from the pER336 plasmid were subjected to gel filtration at various times after mixing. A plot of homodimer concentration (determined from peak area) *versus* time after mixing fits a first order exponential (Fig. 13); the rate constant for dissociation was determined to be $0.60 \pm 0.14 \text{ hr}^{-1}$, corresponding to a half-life of 1.2 hours.

The effects of ligand binding on the dimer dissociation were also examined. For these experiments the protein was pre-equilibrated with saturating amounts of estradiol or 4-hydroxytamoxifen. These results, also shown in Fig. 13, are

summarized in Table I. The data suggest that presence of estradiol significantly decreased the rate of dissociation of the receptor dimer. The antagonist ligand *trans*-4-hydroxytamoxifen was also found to significantly decrease the rate of dissociation, to an even greater degree than estradiol.

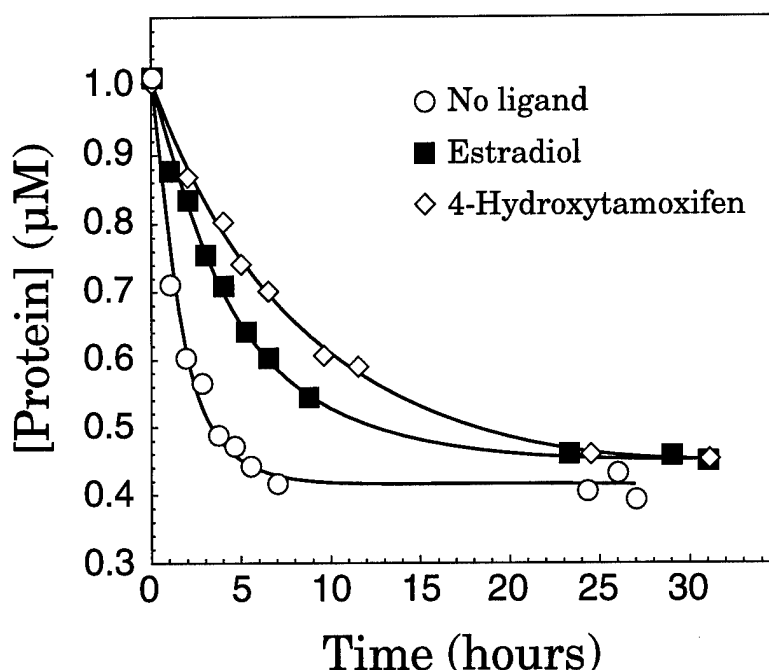


Figure 13. Kinetics of homodimer dissociation. The homodimer concentration (determined from the peak area corrected for protein extinction coefficient) was plotted against time after mixing. For clarity, only the pER336 fusion protein homodimer concentration is shown; HBD peptide homodimer yielded similar kinetics. The curves represent non-linear regression fits to an exponential rate equation. ○, absence of ligand; ■, presence of estradiol; and ◇, presence of 4-hydroxytamoxifen.

The kinetics of dimer dissociation was also tested for pER304, pER330, and pER335 constructs; the results of these experiments are also summarized in Table I.

The results presented in Table I suggest that relatively subtle changes in the N-terminal residues of the HBD have significant effects on the rate of dimer dissociation. Thus, pER336 and pER304, differing by the replacement of Ser-305 with a Glu, have $t_{1/2}$ for dissociation that differ by a factor of 2.

The estrogen receptor mutations that have previously been shown to affect dimerization (9) were all located in the C-terminal region of the HBD (positions 503, 507, 514), and the dimer contact residues in the RXR crystal structure are also located in the C-terminal half of the HBD for that protein (see Fig. 2). This suggests that either additional dimer contacts are in fact present in the N-terminus of the HBD or that the N-terminal mutations tested in these experiments affect the

conformation of the dimer interface without being directly involved in dimer contacts.

Table I.
Summary of Dimer Dissociation Kinetic Experiments

Protein	Ligand	$t_{1/2}$ (hours)	Fold change
pER336 (Gly+300-551 S305E)	None	1.2 ± 0.3	--
	Estradiol	3.7 ± 0.6	3.0 <i>vs.</i> no ligand
	4-Hydroxytamoxifen	5.3 ± 1.4	4.4 <i>vs.</i> no ligand
pER304 (Gly+300-551)	None	2.4 ± 0.4	2.0 <i>vs.</i> pER336
pER335 (Gly+306-551)	None	1.4 ± 0.2	1.2 <i>vs.</i> pER336
	Estradiol	4.7 ± 0.5	3.3 <i>vs.</i> no ligand
	4-Hydroxytamoxifen	6.3 ± 0.7	4.4 <i>vs.</i> no ligand
pER330 (Gly-Arg+305-551)	None	2.2 ± 0.3	1.8 <i>vs.</i> pER336
	Estradiol	7.3 ± 1.2	3.4 <i>vs.</i> no ligand
	4-Hydroxytamoxifen	11.6 ± 2.1	5.4 <i>vs.</i> no ligand

The effect of ligand binding appears to be an increase in $t_{1/2}$ of dissociation. For all HBD peptides tested thus far, the effect of estradiol binding is a ~3-fold increase, and of 4-hydroxytamoxifen is a 4- to 5-fold increase in $t_{1/2}$ compared to the same peptide in the absence of ligand. This suggests that altered structure in the N-terminal region of the HBD has at most limited effect on the conformational changes within the HBD that occur due to ligand binding.

The change in dissociation kinetics also suggests that ligand binding results in a conformational change in the HBD that affects the dimer interface. The fact that the antagonist 4-hydroxytamoxifen also decreases the rate of dissociation implies that it induces conformational changes in the dimer interface similar to those of estradiol. The increase in half-life suggests that one role of ligand binding is to increase the kinetic stability of the estrogen receptor dimer-ligand complex. One effect of this may be to increase the time available for the receptor dimer to interact with the other proteins of the transcription initiation complex.

Cooperativity of Estradiol Binding

The full-length estrogen receptor has been shown to exhibit cooperative ligand binding (30,31). In the course of studying ligand binding by the isolated HBD, it has become obvious that the HBD peptides also exhibit cooperativity. The previous Progress report discussed analysis of cooperativity for the pER304 and pER330 HBD peptides.

Thus far, all of the constructs that exhibit estradiol binding also exhibit positive cooperativity for estradiol binding. For the peptides that have been more extensively characterized, the cooperativity becomes apparent within a narrow range of protein concentration. Figure 13 shows the transition from non-cooperativity (Hill coefficient = 1.0) to positive cooperativity (Hill coefficient = 1.4 to 1.6) for two different constructs. Two properties can be extracted from the data illustrated in Fig. 14: the maximal Hill coefficient observed for the peptide, and the mid-point peptide concentration at which the transition from non-cooperative to cooperative behavior occurs. Table II summarizes the results for the HBD constructs that have been extensively characterized.

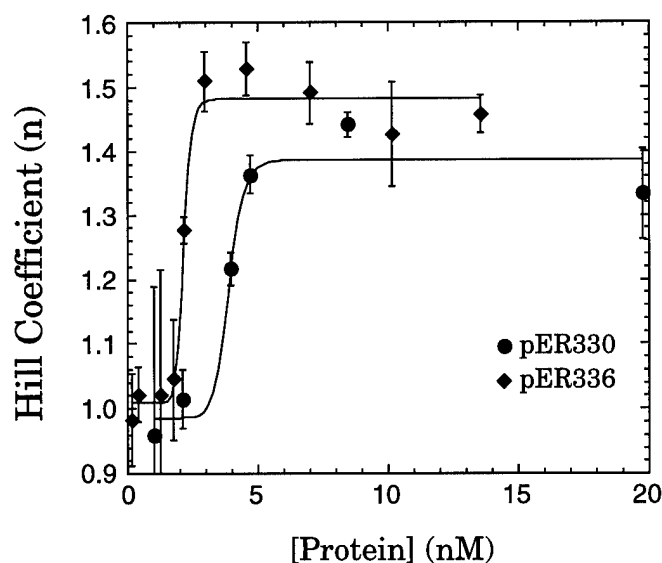


Figure 14. Variation in Hill coefficient for estradiol binding with HBD peptide concentration. Each data point represents the mean \pm SEM from 3 to 9 experiments.

The various HBD peptide constructs all exhibited positively cooperative estradiol binding, with a maximal Hill coefficient of ~ 1.5 . Cooperativity of estradiol binding to the estrogen receptor has been most extensively studied using the protein from calf uterine cytosol (30,35), and the maximal Hill coefficient cited in these reports is ~ 1.6 at a concentration of ~ 5 nM receptor. Using recombinant full-length human estrogen receptor expressed in *Sf9* insect cells, Obourn *et al.* (31) also found

a maximal Hill coefficient of ~1.6; their data suggest that at receptor concentrations below ~1 nM, the receptor does not exhibit cooperativity, while maximal cooperativity is observed at 10 to 20 nM receptor concentration. We observe a transition from non-cooperative to cooperative behavior for the isolated HBD fragments in the range from 1 to 4 nM depending on the peptide, suggesting that the HBD peptide undergoes the conformational changes required for cooperativity in a manner comparable to the full-length protein. One interpretation of these results is that dimer formation occurs over this peptide concentration range in the presence of estradiol. Thus, at concentrations significantly below the mid-point concentration, the HBD peptide exists as a monomer, while at concentrations greater than about twice the mid-point concentration, the HBD peptide is predominantly present as a dimer which exhibits a cooperative interaction with estradiol.

Table II.
Cooperativity of Estradiol Binding by Different HBD Peptides

Peptide	Mid-point Peptide Concentration (nM)	Maximal Hill Coefficient
pER336 (Gly+300-551 S305E)	2.1 ± 0.1	1.51 ± 0.06
pER304 (Gly+300-551)	1.4 ± 0.5	1.47 ± 0.11
pER335 (Gly+306-551)	0.8	1.56
pER330 (Gly-Arg+305-551)	3.8 ± 0.23	1.40 ± 0.07

The results presented in Table II suggest that, as with the dimer dissociation kinetics, mutations near the N-terminus of the HBD may affect the dimer interactions affinity. It is possible that the cooperativity experiments may allow an indirect measurement of the dimer interaction affinity. It should be noted that this may or may not correlate with the rate of homodimer dissociation measured in the kinetic experiments using size-exclusion chromatography, since the dimer interaction affinity is the ratio of the dissociation rate constant (measured in the kinetic experiments) and the association rate constant (which is not directly measurable). Based on the fact that the pER330 peptide has the lowest apparent dimer interaction affinity (Table II), but one of the slower rates of dissociation (Table I), it is likely that the dissociation rate constant and association rate constant vary independently for the different peptides.

Crystallization Attempts

One of the major goals of this project is to obtain structural information for the HBD using X-ray crystallographic analysis; this would be particularly useful for rational drug design. In order to determine the structure of the protein using this method, it is necessary to produce large amounts of homogeneous protein and to find conditions under which the protein will crystallize. The first of these goals has been met; the second has proven rather difficult. Several hundred milligrams of a number of different fusion proteins have been produced and apparently homogeneous cleavage of two of these (pER330 and pER336) in large quantities has been achieved.

In attempting to crystallize a protein it is useful to have stock solution of a fairly high concentration of the protein of interest (typically ~20 mg/ml or greater). All of the fusion proteins tested can be concentrated to ~40 mg/ml. However, the isolated HBD peptide is hydrophobic and attempts to concentrate the HBD peptide by standard ultrafiltration (Amicon pressure cell, or Centricon centrifugation ultrafilters) resulted in losses of large amounts of material due to precipitation of the peptide and maximal HBD peptide concentrations of ~3 mg/ml. More recently, use of Centriprep concentrators (purchased from Amicon) in which the ultrafiltration membrane is present in a plunger (Fig. 15), have resulted in higher protein concentrations (~12 mg/ml for pER330 HBD peptide; ~18 mg/ml for pER336 HBD peptide), and smaller losses of material (~10-20% instead of ~50%) due to denaturation or adsorption to the filtration membrane.

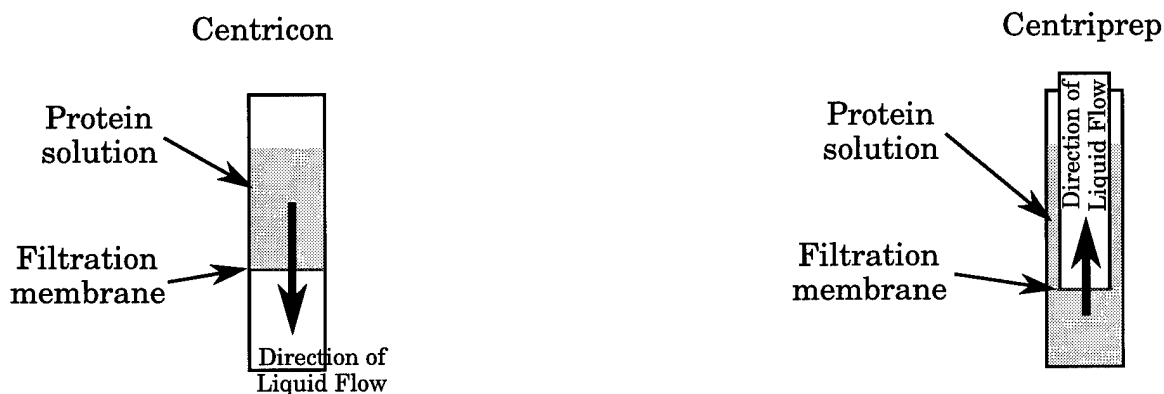


Figure 15. Different types of ultrafiltration devices. In both cases the force induced by centrifugation is oriented downward; in the case of the Centriprep, the liquid flow is in the opposite orientation and, probably as a result, higher concentrations and smaller losses of HBD peptides were obtained.

The Hampton Research screening kits have been used to attempt to find crystallization conditions for the pER304, pER330, and pER336 HBD peptides and their fusion proteins, both in the presence and absence of estradiol. The screening kits contain a total of 98 different solutions covering a wide range of conditions, and are referred to as a sparse matrix screen. None of the conditions tried has yielded crystals; many of the conditions resulted in precipitation of the protein. Decreasing the precipitant concentration for conditions that resulted in precipitation also did

not yield crystals.

A variety of other expression plasmids have been constructed (see next section); as protein becomes available, it will be tested for crystal formation. In addition, constructs expressing smaller fragments of the HBD are planned in the hopes that these will exhibit a lesser tendency to aggregate non-specifically.

Mutant Construction and Preliminary Analysis

A large number of additional constructs have been produced. These are summarized in the Appendix. The plasmids fall into five general categories: 1) those intended to modify the N-terminal region of the HBD (initially a result of the search for improved cleavage, and more recently as a result of the finding that the N-terminus is involved in dimer interactions); 2) tryptophan mutants (intended to allow characterization of the effects of ligand binding on fluorescence); 3) mutations with altered C-termini; 4) mutants intended to examine the dimer interactions; and 5) mutants produced as a result of artifacts during construction of other plasmids.

It is possible to measure estradiol binding in crude lysates from small (~20 ml) cultures; an *E. coli* lysate that lacks an HBD constructs does not exhibit detectable estradiol binding. A simple screening assay, in which estradiol binding is measured using serial dilutions of crude lysate therefore allows a rapid determination of the ability of a new construct to bind estradiol. Crude lysate can also be used as a source of material for estimation of K_d for estradiol, although this is somewhat more difficult due to the presence of cooperative estradiol binding. The Appendix lists a number of different constructs and brief comments on their properties.

The N-terminal region mutants include pER304, pER330, pER331, pER333, pER335, and pER336, which have been described above. In addition, pER337, which was intended to express Gly-Arg+315-551 has been constructed. The fusion protein expressed from the pER337 plasmid binds estradiol, but is unstable, and exhibits a marked tendency to aggregate and precipitate during attempts at purification. This suggests that the minimum HBD starting residue is somewhere between 305 and 315 (Gly+306-551 is stable, and Gly-Arg+315-551 is not). Constructs beginning between these locations are under construction.

We have shown that the HBD peptide exhibits fluorescence, and that this fluorescence is quenched by 4-hydroxytamoxifen (see original application). We proposed to examine the effect of tryptophan mutations on the properties of the protein and on the effects of ligand binding. Figure 2 shows the locations of the Trp residues. Trp-360 and Trp-383 are conserved in RXR- α ; all of the Trp residues are conserved in all of the estrogen receptor sequences known, and among all of the human steroid receptors. Mutation of the Trp to Phe was chosen since Phe has negligible fluorescence and is otherwise fairly similar to Trp. The single mutants, W383F and W393F, produced stable proteins; however, W360F did not. While it was possible to measure estradiol binding for the W360F fusion protein (K_d = ~1.5 nM, suggesting somewhat reduced affinity for estradiol), the protein denatured during

the purification procedure. Mutation of Trp-360 to Tyr appears to result in a more stable protein. The full characterization of the single and multiple Trp mutants is in progress.

The full-length estrogen receptor ends at amino acid 595. In attempting to express protein that extends to the C-terminus of the native protein, it was discovered that the additional peptide destabilized the protein. Western blot analysis of whole cells using anti-serum raised against the HBD indicated that the majority of the protein was truncated to a size similar to that produced by pER03 (MBP+linker+301-567). Seielstad *et al.* (12) report that their construct was also cleaved by *E. coli* proteases after positions 569 or 571. In addition to the proteolysis, fusion proteins from pER332 and an earlier construct, pER05, are only produced in small amounts, suggesting that the 3'-end of the mRNA or the C-terminus of the protein for these constructs inhibits expression or enhances degradation of the protein. As mentioned above, the shorter construct, pER334, intended to end at position 534, appears to express normally, but the alteration in the C-terminal length changes the conformation such that hydroxylamine no longer cleaves at the correct site.

Estrogen receptor HBD residues previously reported to be involved in dimerization include 503, 507, and 514 (9); mutations of Ile-514 were reported to abolish estradiol binding. Initial experiments were intended to replicate these results, and the R503A and L507R mutations used by Fawell *et al.* (9) were constructed. In contrast to their finding that these mutations had little effect on estradiol binding, these mutants were found to be unstable. In most attempts, L507R expresses little protein; in one case enough protein was produced to assay for estradiol binding, which was found to be fairly close to normal affinity ($K_d \sim 0.6$ nM). The L507R mutant is likely to prove too unstable to allow purification of significant amounts of active protein. Examination of the sequence alignment with RXR- α (Fig. 2), suggests that Leu-507 may actually be pointing inward toward the hydrophobic core of the HBD monomer; the alignment suggests that it may be the adjacent residues (Gln-506 and Leu-508) that form dimer contacts. Mutation of these residues, and of the potential dimer contact Lys-492 (which is also a Lys in the RXR- α sequence) are in progress.

In the course of sequencing some of the plasmids discussed, it was found that in addition to the intended construct, a number of artifacts were produced. The majority of these were the result of errors introduced by *Taq* polymerase during PCR-based sub-cloning or mutagenesis. However, during attempts to construct a K492Q mutant by Unique Site Elimination mutagenesis, at least 5 different deletion mutations were observed. The mutagenesis technique requires the use of a DNA-repair (*mutS*) deficient strain of *E. coli*; for the K492Q mutation, a nearby unique *Stu* I site used for selection (the same primer contained the K492Q mutation and a silent mutation that disrupted the *Stu* I site). It seems likely that this resulted in a high probability for deletions within this region. (In two of the mutant plasmids, the deletion of 96 bases, coding for amino acids 474-505 and 483-514, left the remainder of the coding region in frame. These plasmids produced protein of the predicted size, but estradiol binding by the fusion protein was below the limit of detection in the radioreceptor assay.)

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Appendix Partial List of Plasmids Constructed

Plasmid	Insert Properties			Comments
	Start	End	Mutation	
pER08	301	551	None	Not designed to be hydroxylamine cleavable.
pER304	Gly+ 300	551	None	Somewhat heterogeneous cleavage by hydroxylamine $K_d = 0.25 \pm 0.14$ nM dissociation $t_{1/2} = 2.4 \pm 0.4$ hr
pER336	Gly+ 300	551	S305E	Homogeneous cleavage $K_d = 0.18 \pm 0.03$ nM dissociation $t_{1/2} = 1.2 \pm 0.3$ hr
pER330	Gly- Arg+ 305	551	None	Homogeneous cleavage $K_d = 0.20 \pm 0.04$ nM dissociation $t_{1/2} = 2.2 \pm 0.3$ hr
pER335	Gly+ 306	551	None	Actual insert begins at estrogen receptor codon 300; contains S305G mutation to improve cleavage; Somewhat heterogeneous cleavage by hydroxylamine (additional cleavage site within MBP, in spite of mutation of MBP Asn-368) $K_d = 0.19 \pm 0.08$ dissociation $t_{1/2} = 1.4 \pm 0.2$ hr
pER337	Gly- Arg+ 315	551	None	Unstable; fusion protein binds estradiol, but rapidly loses activity during purification.
pER331	Gly+ 306	551	None	Actual insert begins at estrogen receptor codon 279; contains S305G mutation to improve cleavage; Somewhat heterogeneous cleavage by hydroxylamine (additional cleavage site within MBP)
pER333	Gly+ 306	551	None	Actual insert begins at estrogen receptor codon 300; contains S305G mutation to improve cleavage; Somewhat heterogeneous cleavage by hydroxylamine (additional cleavage site within MBP)
pER03	301	567	None	Not designed to be hydroxylamine cleavable.
pER05	279	595		Not designed to be hydroxylamine cleavable. Poor expression; proteolytic degradation occurs within cells.
pER332	Gly- Arg+ 305	595	None	Poor expression; proteolytic degradation occurs within cells.

pER334	Gly- Arg+ 305	534	None	Heterogeneous cleavage Fusion protein $K_d = 0.14$
pER306	Gly+ 300	551	W360F	Somewhat unstable Fusion protein $K_d = 1.56 \pm 0.21$
pER316	Gly+ 300	551	W360Y	Fusion protein $K_d = 0.52 \pm 0.01$
pER307	Gly+ 300	551	W383F	$K_d = 0.39$
pER308	Gly+ 300	551	W393F	$K_d = 0.41$
pER310	Gly+ 300	551	W360F, W383F	Constructed and sequenced (expected to be unstable).
pER311	Gly+ 300	551	W360F, W383F	Constructed and sequenced (expected to be unstable).
pER312	Gly+ 300	551	W383F, W393F	High level of estradiol binding in screening assay.
pER313	Gly+ 300	551	W360F, W383F, W393F	Unstable
pER318	Gly+ 300	551	W360Y, W383F, W393F	Constructed and sequenced.
pER314	Gly+ 300	551	R503A	Unstable
pER315	Gly+ 300	551	L507R	Low level of protein expression (unstable?) Fusion protein $K_d = 0.6$
pER317	Gly+ 300	551	S305E, C447W	Fusion protein $K_d = \sim 1.6$ (low but detectable activity in screening assay)
pER305	Gly+ 300	551	G366S	Fusion protein $K_d = 0.45$
pER11	279	567	A361V	Fusion protein $K_d = 0.32$
pER04	Gly+ 300	567	S433P	Estradiol binding detectable but very low ($\sim 100\times$ below active constructs in screening assay).
pER370	Gly+ 300	551	$\Delta 483-514$	Extremely low activity in screening assay.
pER371	Gly+ 300	551	$\Delta 474-505$	Extremely low activity in screening assay.